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Request for grant of a patent

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1. Your reference	AHB/BP5739313		
2. Patent application number *(The Patent Office will fill in this part)*	**9907414.8**		31 MAR 1999
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED CAMBRIDGE HOUSE 6-10 CAMBRIDGE TERRACE REGENT'S PARK LONDON NW1 4JL UNITED KINGDOM		3784150002
Patents ADP number *(if you know it)*			
If the applicant is a corporate body, give the country/state of its incorporation	GB		
4. Title of the invention	IMPROVEMENTS RELATING TO PRODRUGS		
5. Name of your agent *(if you have one)*	MEWBURN ELLIS		
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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application		Number of earlier application	Date of filing *(day / month /year)*
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11. I/We request the grant of a patent on the basis of this application.

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12. Name and daytime telephone number of person to contact in the United Kingdom ADRIAN H BRASNETT 0171 240 4405

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IMPROVEMENTS RELATING TO PRODRUGS

This invention relates to prodrugs, their use in therapy and a process for their preparation.

Over the years, many cytotoxic compounds have been discovered which are of potential use in cancer chemotherapy. Nitrogen mustards from one important family of such cytotoxic compounds. The clinical use of cytotoxic compounds in general and nitrogen mustards in particular has been limited because of the poor selectivity in the cytotoxic effect between tumour cells and normal cells.

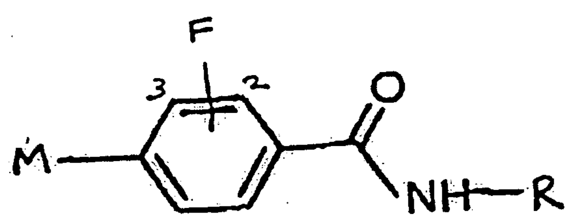
One approach to overcome this problem has involved the development of so-called prodrugs which are derivatives of the cytotoxic drug, often relatively simple derivatives, whose cytotoxic properties are considerably reduced compared to those of the parent drugs. Numerous proposals have been made for the administration of such prodrugs to patients under regimes whereby the prodrug is only converted to the cytotoxic drug in the region of the intended site of action.

A variety of systems exist for delivery of the enzyme. One such system is described in WO88/07378, and involves conjugating the enzyme to an antibody specific for a tumour marker, delivering the antibody enzyme conjugate to a patient, allowing the conjugate to localise, and then delivering the prodrug to the patient. This system is referred to as antibody-directed enzyme prodrug therapy" (ADEPT).

Various other proposals for delivery of the enzyme to the desired site of action exist, including gene-directed enzyme prodrug therapy (GDEPT), in which a vector such as a viral vector carrying a gene encoding the prodrug-converting enzyme is delivered to cells at the desired site of action (Huber et al, Proc. Natl. Acad. Sci. USA (1991) 88, 8039). GDEPT is also

described in WO96/03151, the disclosure of which is incorporated herein by reference. A further alternative system is to provide a ligand, generally a naturally occurring polypeptide whose biological role involves its binding to a cognate receptor on the surface of the cell, conjugated to the prodrug-activating enzyme. This system, LIDEPT, is described in WO/97/26918, where VEGF is particularly exemplified as an example of a ligand.

One class of prodrugs suggested for use in the above systems is that of prodrugs of nitrogen mustard compounds. Benzoic acid nitrogen mustards are bifunctional alkylating agents, and a variety of prodrugs of such compounds are described in the art. One class of such prodrugs comprise a protecting group which may be removed by the action of a carboxypeptidase enzyme, such as bacterial carboxypeptidase G, including such enzymes available from Pseudomans species, such as carboxypeptidase G2 (CPG2). This enzyme, and prodrugs which may be activated by it, are described in Springer <u>et al</u>., J. Medicament. Chem., (1990) <u>33</u>, 677-681 and Springer <u>et al</u>., Anti-Cancer Drug Design (1991) <u>6</u>, 467-479, WO88/07378, WO94/25429 and WO96/22277.



(X)

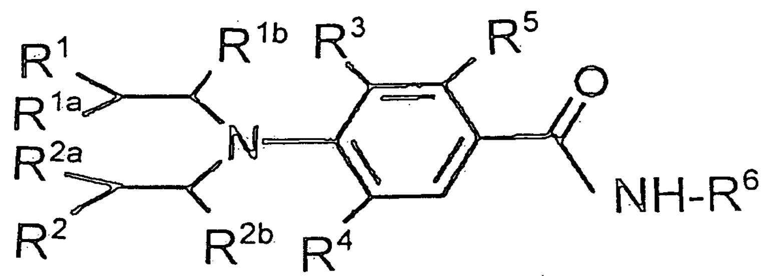
WO94/25429 in particular describes 2-fluoro ring substituted and 3-fluoro ring substituted benzoic acid nitrogen mustards of general formula (X):

wherein R-NH is the residue of an α-amino acid R-NH_2 or oligopeptide R-NH_2, and M is a nitrogen mustard group of the

formula $(LCH_2CH_2)(YCH_2CH_2)N-$, wherein Y and L, which may be the same or different in a molecule, are leaving groups.

The prodrug is converted into the active drug by cleavage of the amide bond between the residue of the α-amino acid $R-NH_2$ or oligopeptide $R-NH_2$ and the residue of the benzoic acid nitrogen mustard.

Thus although a number of nitrogen mustard prodrugs exist in the art, there is a continuing need for improved prodrugs, which can provide enhanced efficacy, for example by having an improved ratio of toxicity between prodrug and active drug form, better half-life or more potency. Accordingly, in a first aspect the present invention provides a compound of general formula (I)



wherein

R^1 is Cl, Br, I, CH_3SO_2O, or OSO_2phenyl (wherein phenyl is optionally substituted with 1, 2, 3, 4 or 5 substituents independently selected from C_{1-4} alkyl, halogen, -CN or $-NO_2$);

R^2 is Cl, Br, I, CH_3SO_2O, or OSO_2phenyl (wherein phenyl is optionally substituted with 1, 2, 3, 4 or 5 substituents independently selected from C_{1-4} alkyl, halogen, -CN or $-NO_2$);

R^{1a} and R^{2a} each independently represent hydrogen, C_{1-4} alkyl or C_{1-4} haloalkyl;

R^{1b} and R^{2b} each independently represent hydrogen, C_{1-4} alkyl or C_{1-4} haloalkyl;

R^3 is F, Cl, Br, I, $OCHF_2$, C≡CH, OCF_3, CH_3, CF_3, SF_5, SCF_3 or

CF_2CF_3;

R^4 is F, Cl, Br, I, $OCHF_2$, C≡CH, OCF_3, CH_3, CF_3, SF_5, SCF_3 or CF_2CF_3;

R^5 is H or F;

R^6 is a group -CH(Z)-C(O)-OR^7 wherein

R^7 is hydrogen, t-butyl or allyl; Z is a group -V-W where V is -CH_2-T- in which T is -CH_2-, -O-, -S-, -(SO)-, or -(SO_2)- (provided that when V has sulphur or oxygen as its second atom, W is other than -COOH) and said group V is optionally further carrying one or two substituents Q^1 and/or Q^2 on carbon;

wherein Q^1 and Q^2 each independently represents C_{1-4} alkyl or halogen; or, when Q^1 and Q^2 are bonded to adjacent carbon atoms, Q^1 and Q^2 together may additionally represent a C_3-C_4 alkylene radical optionally substituted with 1, 2, 3 or 4 substituents independently selected from the group consisting of C_{1-4} alkyl and halogen: and W represents

(1) COOH,

(2) -(C=O)-O-R^8 wherein R^8 represents a C_{1-6} alkyl, allyl, C_{3-6} cycloalkyl or aryl (as defined in 3 below) group;

(3) -(C=O)-NR^8R^9 wherein R^8 and R^9 each independently represent hydrogen or a C_{1-6} alkyl, C_{3-6} cycloalkyl, phenyl, heteroaryl linked to N via carbon or C_{7-9} aralkyl group wherein heteroaryl is a 5 or 6 membered ring containing 1 to 3 heteroatoms selected from the group consisting of nitrogen and sulphur;

the phenyl moiety *per se*, the heteroaryl moiety and the aryl moiety of the arylkyl group may be substituted on carbon with 1-4 substituents selected from the group consisting of -COOH, -OH, -NH_2, -CH_2-NH_2, -$(CH_2)_{1-4}$-COOH, tetrazol-5-yl and -SO_3H and the alkyl moiety may optionally carry a methyl group;

(4) -SO_2NHR^{10} wherein R^{10} is as defined for R^8 but may additionally represent -CF_3, -CH_2CF_3 or phenyl as defined above;

(5) SO_3R^{11} in which R^{11} represents H, C_{1-6} alkyl or C_{3-6}

cycloalkyl,

(6) $PO_3R^{11}R^{11}$ (wherein the R^{10} radicals, which may be the same or different, are as herein defined)

(7) a tetrazol-5-yl group:

(8) $-CONH-SO_2R^{12}$ in which R^{12} represents

(a) C_{3-7} cycloalkyl;

(b) C_{1-6}-alkyl optionally substituted with substituents selected from the group consisting of aryl as defined below, C_{1-4}-alkyl, CF_3 or halogen; and

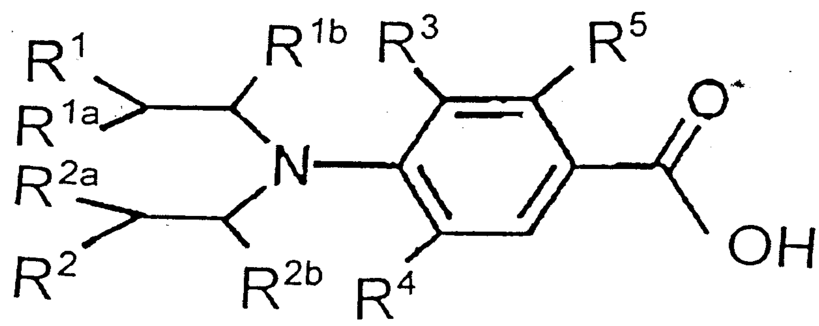
(c) perfluoro-C_{1-6} alkyl; wherein aryl is phenyl or phenyl having 1-5 substituents wherein the substituents are selected from the group consisting of halogen, $-NO_2$, $-CF_3$, C_{1-4} alkyl, C_{1-4} alkoxy, $-NH_2$, $-NHCOCH_3$, $-CONH_2$, $-OCH_2COOH$, $-NH(C_{1-4}$-alkyl), $-N(C_{1-4}$-alkyl$)_2$, $-NHCOOC_{1-4}$-alkyl, -OH, -COOH, -CN and $-COOC_{1-4}$alkyl; and

(9) -M-Het wherein M represents S, SO or SO_2 and Het represents a 5 or 6 membered heterocyclic aromatic ring linked to M via a carbon atom of the aromatic ring, said aromatic ring containing 1, 2, 3 or 4 heteroatoms selected from the group consisting of O, N and S said aromatic ring optionally being substituted on carbon atoms of the ring with 1, 2, 3 or 4 substituents selected from the group consisting of -OH, -SH, -CN, $-CF_3$, NH_2 and halogen;

and physiologically acceptable derivatives of the compound of formula (I).

The compound of formula (I) is a prodrug which may be converted into the corresponding active drug by cleavage of the amide bond between the residue of R^6-NH2 and the benzoic acid nitrogen

mustard residue. The active drug has the formula (II):



wherein R^1, R^{1a}, R^{1b}, R^2, R^{2a}, R^{2c}, R^3, R^4 and R^5 are defined above. The compound of formula (II) is also novel and forms part of the invention.

Preferred compounds of formulae (I) and (II) are those in which R^1 and R^2 are I, R^{1a}, R^{1b}, R^{2a}, R^{2b} are all hydrogen, the remaining groups being defined as above.

Other preferred compounds of formulae (I) and (II) are:

1: Compounds of the formulae (I) and (II) in which R^3 and R^4 are both be F, and R^5 is H or F, the remaining groups being as defined above.

2: Compounds of the formula (I) in which, R^7 is preferably H or t-butyl, and Z is a group $-CH_2-T-C(O)-OR^8$ as defined above. More preferably, T is $-CH_2-$ and R^8 is hydrogen, t-butyl or allyl.

3: Compounds of the formulae (I) and (II) in which R^1 and R^2 are iodo and at least R^3 and R^4 are fluoro. These are particularly potent and such compounds comprise a preferred aspect of the invention. In this aspect of the invention, the groups R^5 and R^6 may have any of the values

as defined above although in a most preferred aspect R^5 is H or F and R^6 is a group in which R^7 is preferably H or t-butyl, and Z is a group $-CH_2-T-C(O)-OR^8$ as defined above but more preferably T is $-CH_2-$ and R^8 is hydrogen or t-butyl.

4: Compounds of 1-3 above in which R^{1a}, R^{2a}, R^{1b}, R^{2b} are all hydrogen.

Examples of prodrugs of the invention are:

[3,5 - Difluoro -4-[bis(2-iodoethyl) amino] benzoyl]-L-glutamic acid;

[3,5 - Difluoro-4-[bis(2-chloroethyl)amino]benzoyl]-L-glutamic acid;

[3,5 - Difluoro-4-[bis(2-bromoethyl)amino]benzoyl]-L-glutamic acid;

4-[Bis(2-chloroethyl)amino] - 2, 3, 5-trifluorobenzoyl -L-glutamic acid;

and pharmaceutically acceptable salts thereof.

Pharmaceutically acceptable salts include, but are not limited to: base salts, e.g. those derived from an alkali metal (e.g. sodium) or alkaline earth metal (e.g. magnesium), and ammonium salts; and acid addition salts, including hydrochloride and acetate salts.

Compounds of the invention in which R^7 is other than hydrogen and in which R^8 is present on a group $COOR^8$ are usually made as intermediates in the production of compounds in which R^7 and R^8 are both H, which are preferred for use as prodrugs. This is so as to provide protection of the carboxy groups of the compound during synthesis of the nitrogen mustard moiety, as shown in the accompanying reaction schemes and examples. Removal of such protecting groups is illustrated in the

accompanying examples, but also described in WO88/07378 and WO90/02729. Compounds of the invention of this type include:

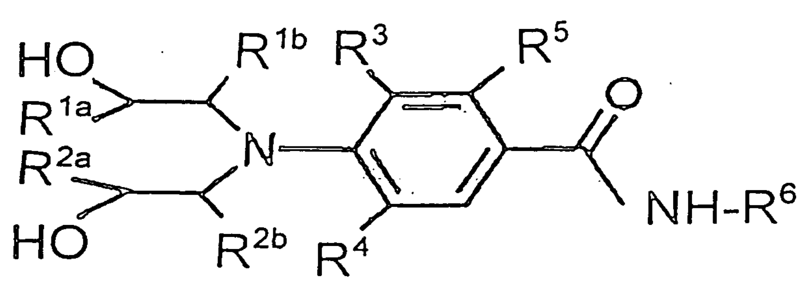
Di-tert-butyl [4-[bis(2-bromoethyl)amino] - 2, 3, 5-trifluorobenzoyl]-L-glutamate;
Di-tert-butyl [4-[bis(2-iodoethyl)amino] - 2, 3, 5-triflurobenzoyl]-L-glutamate;
and pharmaceutically acceptable salts thereof.

In a further aspect, the present invention provides a process including deprotecting a compound of formula (I) wherein one or both of R^7 or R^8 are other than hydrogen. A suitable method is by treatment with an acid, for example in a substantially non-aqueous medium, e.g. at room temperature (e.g. at 15-25°C). Trifluoroacetic acid and formic acid are suitable acids.

The process may include converting the deprotected compound into a pharmaceutically acceptable salt thereof.

Prodrugs of the invention may thus be produced by processes analogous to those described in WO88/07378, WO90/02729 and WO91/03460.

Compounds of the invention in which R^1 and R^2 are mesyl, R^7 is not hydroxy, and Z does not include an unprotected hydroxy group may be made from a compound of formula (III):



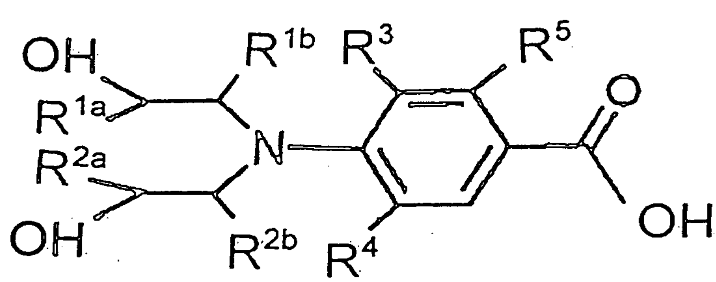
wherein R^6 is a group such that R^7 is not hydroxy and Z does not include an unprotected hydroxy group, by reaction with methane sulphonic anhydride in CH_2Cl_2 and triethylamine at room temperature.

Similarly, compounds of the formula (III) may be reacted with optionally substituted phenyl sulphonic anhydride under similar conditions to provide compounds of the invention in which R^1 and R^2 are optionally substituted OSO_2 phenyl.

The bismesyl compound described above may be converted into other compounds of the invention in which R^7 is OH and Z includes an unprotected hydroxy group by deprotection in TFA as described in the examples herein.

Alternatively, the protected bis mesyl compound may be used to provide further compounds of the invention by treatment with an alkali metal halide (e.g. LiBr, LiCl or NaI) in a suitable solvent (e.g. acetone or N,N-dimethylacetamide) under elvated temperature or under reflux, to provide for substitution of one or both mesyl groups by the appropriate halide. The resulting compound may be deprotected as above.

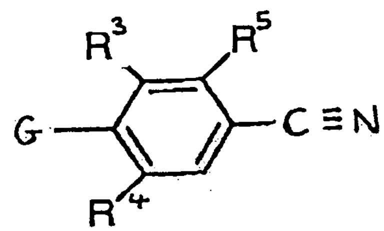
Compounds of the formula (III) may be made by reaction of a compound of formula (IV):



with a compound of formula H_2N-CH(Z)C(O)OR^7 where Z and R^7 are

as defined above in a suitable solvent (e.g. N,N-dimethylformamide) in the presence of diethylcyanophosphate and base.

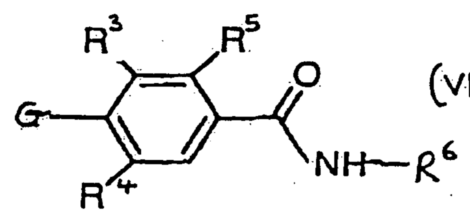
Compounds of the formula (IV) may be prepared in any convenient way. A suitable means is by reaction of a the corresponding nitrile compound with a strong base (e.g. NaOH) in an aqueous solvent such as ethanol under reflux. The nitrile may be obtained by reaction of a compound of the formula (V):



(V)

where G is a group capable of being substituted, such as F or Cl, with diethanolamine or protected diethanolamine in a suitable solvent such as N,N-dimethlyacetamide.

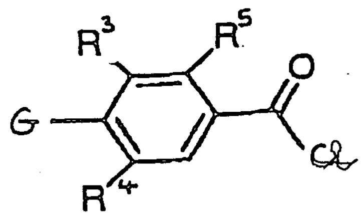
Alternatively the compound of formula (III) where R^3, R^4 and R^5 are all fluoro may be made by reaction of a compound of formula (VI)



(VI)

where R^3, R^4 and R^5 are all fluoro, where R^7 is other than OH, Z does not include an unprotected OH group and G is as defined above, with diethanolamine in a suitable solvent such as N,N-dimethlyacetamide.

The compound of the formula (VI) may be obtained by reaction of a compound of formula (VII):



(VII)

where R^3, R^4 and R^5 are fluoro, with a compound of formula H_2N-$CH(Z)C(O)OR^7$, with Z and R^7 defined as for the compound of formula (VI).

The prodrugs of the present invention are suitable for use in a method of treatment of the human or animal body by therapy, particularly a method of treatment of cancer or other conditions in which selective killing of target cells in the body of a patient is required. The animal may be a non-human mammal. The cancer may be any disease in which there is a neoplastic cell growth, including leukemias and solid tumours. Examples of tumours which may be treated in accordance with the present invention include breast, colorectal and ovarian tumours, as well as pancreatic, melanoma, glioblastoma, hepatoma, small cell lung, non-small cell lung, muscle and prostate tumours.

It will be understood by those of skill in the art that the treatment of a tumour includes any measure taken by a physician to alleviate the effect of the tumour on a patient. Thus, although complete remission of the tumour is a desirable goal, effective treatment will also include any measure capable of achieving partial remission of the tumour as well as a slowing down in the rate of growth of a tumour including metastases. Such measures can be effective in prolonging and/or enhancing the quality of life and/or relieving the symptoms of the

disease.

The present invention thus extends in various aspects to the use of a prodrug of the invention in a method of medical treatment, particularly a method of treatment of cancer; the use of such a prodrug in the manufacture of a composition for the treatment of a disease, disorder or other medical condition, e.g. in the manufacture of a composition for the treatment of cancer; a method of treating a human or animal patient suffering from cancer, the method comprising administering to the patient a prodrug of the invention in combination with an enzyme-prodrug therapy; and a method of making a pharmaceutical composition comprising admixing a prodrug of the invention with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

A prodrug of the invention may be selectively converted to the corresponding active drug by the enzyme component of an enzyme-prodrug therapy such as ADEPT, GDEPT or LIDEPT system. Accordingly, the present invention provides a two component system comprising:

(i) a first component capable of delivering a carboxypeptidase enzyme to the interior or exterior of a target cell or a vector encoding said enzyme to the interior of said cell such that said vector expresses said enzyme in said cell, and
(ii) a prodrug of formula (I) capable of being converted by said enzyme into a drug of formula (II).

It will be apparent to those of skill in the art that the first component may be in the form of an antibody or ligand conjugated to the enzyme, or a vector capable of expressing a

carboxypeptidase enzyme, or indeed any other suitable form for delivery of the enzyme to the cell such that activation of a prodrug may occur selectively at the site of the cell. Such means are well known and described as such in the prior art mentioned above, which is incorporated herein by reference.

The term "antibody" should be construed as covering antibody derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimics that of an antibody enabling it to bind an antigen or epitope. Thus the term includes monoclonal and polyclonal antibodies, as well as antibody fragments capable of binding an antigen such as Fab fragments, Fv fragments consisting of the VL and VH domains of a single arm of an antibody or single chain Fvs in which said domains are linked by a polypeptide linker.

Examples of suitable ligands include vascular endothelial growth factor (VEGF), epidermal growth factor, EGF, (which binds to epidermal growth factor receptor), heregulin and c-erbB2 ligand. The known VEGF receptors are tyrosine kinases called flt-1 and flk-1, which are specifically expressed in endothelial cells of the tumour and in the border between the tumour and normal tissue. EGFR and c-ErbB2 are expressed in a number of tumour types.

For GDEPT, a wide variety of vectors are available. These include those which are based upon a retrovirus. Such vectors are widely available in the art. Huber et al (ibid) report the use of amphotropic retroviruses for the transformation of hepatoma, breast, colon or skin cells. Culver et al (Science (1992) <u>256</u>; 1550-1552) also describe the use of retroviral vectors in GDEPT. Such vectors or vectors derived from such vectors may also be used. Other retroviruses may also be used

to make vectors suitable for use in the present invention. Such retroviruses include rous sarcoma virus (RSV). The promoters from such viruses may be used in vectors in a manner analogous to that described above for MLV.

EP-A-415 731 describes molecular chimeras comprising a promoter which may be activated in a tumour cell operably linked to a heterologous gene encoding an enzyme capable of converting a prodrug into a cytotoxic agent. Such molecular chimeras may be used to express carboxypeptidase in tumour cells in order to activate prodrugs of the invention. EP-A-415 731 describes incorporation of such molecular chimeras into viral vectors, e.g. adenoviral or retroviral vectors. Such viral vectors may also be adapted for utilization in the present invention.

Other recombinant viral vector delivery systems are described in WO91/02805, WO92/14829, WO93/10814, WO94/21792, WO95/07994, WO95/14091 and WO96/22277, the disclosures of which are incorporated herein by reference. Methods for producing vector delivery systems based on the above-mentioned disclosures may be used to deliver vectors encoding the activating enzyme (e.g. carboxypeptidase) to target cells.

Englehardt et al (Nature Genetics (1993) 4; 27-34) describes the use of adenovirus based vectors in the delivery of the cystic fibrosis transmembrane conductance product (CFTR) into cells, and such adenovirus based vectors may also be used in accordance with the present invention. Vectors utilising adenovirus promoter and other control sequences may be of use in delivering a system according to the invention to cells in the lung, and hence useful in treating lung tumours.

Vectors encoding the carboxypeptidase may be made using recombinant DNA techniques known per se in the art. The

sequences encoding the enzyme may be constructed by splicing synthetic or recombinant nucleic acid sequences together, or modifying existing sequences by techniques such as site directed mutagenesis. Reference may be made to "Molecular Cloning" by Sambrook et al (1989, Cold Spring Harbor) for discussion of standard recombinant DNA techniques. In general, the vector may be any DNA or RNA vector used in GDEPT therapies.

The carboxypeptidase will be expressed from the vector using a promoter capable of being expressed in the cell to which the vector is targeted. The promoter will be operably linked to the sequences encoding the enzyme and its associated sequences.

Suitable promoters include viral promoters such as mammalian retrovirus or DNA virus promoters, e.g. MLV, CMV, RSV and adenovirus promoters. Preferred adenovirus promoters are the adenovirus early gene promoters. Strong mammalian promoters may also be suitable. An example of such a promoter is the EF-1α promoter which may be obtained by reference to Mizushima and Nagata ((1990), Nucl. Acids Res. 18; 5322). Variants of such promoters retaining substantially similar transcriptional activities may also be used.

The c-erbB2 proto-oncogene is expressed in breast tissues at low levels and in a tissue restricted manner. In some tumour states, however, the expression of this protein in increased, due to enhanced transcriptional activity. Notable examples of this are breast tissue (about 30% of tumours), ovarian (about 20%) and pancreatic tumours (about 50-75%). In such tumours where expression of c-erbB2 is increased due to enhanced transcription or translation, the c-erbB2 promoter may be used to direct expression of the activating enzyme in a cell specific manner. The specificity of GDEPT may be increased

since transfection of normal cells by a vector with a c-erbB2 promoter will provide only very limited amount of enzyme or none and thus limited activation of prodrug. The use of the c-erbB2 promoter and homologous promoters in GDEPT is more fully described in WO96/03151.

A carboxypeptidase enzyme of the invention will be any suitable carboxypeptidase enzyme capable of cleaving the glutamic acid or glutamic acid derivative moiety of compounds of the Formula (I). Preferred carboxypeptidase enzymes are bacterial carboxypeptidases, particularly carboxypeptidase G enzymes including CPG1, 2 or 3. A pseudomonad carboxypeptidase enzyme is described in Levy and Goldman, J. Biol. Chem., 1967, 12, 2933-2938. CPG1 is described by Cornell and Charm, Biotechnology and Bioengineering, Vol XVIII, 1976, 1171-1173. CPG2 is described in WO88/07378 and CPG3 is described by Yasuda *et al*, Bull. Mukogawa, Women's Univ. Nat. Sci, 1994, 42, 63-66. Although naturally occurring carboxypeptidase enzymes are preferred as a matter of convenience, synthetic variants of such enzymes may be made. For example, WO96/03515 described synthetic variants in which one or more of the glycosylation motifs (Asn-Xaa-Ser/Thr) (where Xaa is any amino acids) are altered to prevent glycosylation. For example, within the primary amino acid sequence of CPG2, there are three such consensus motifs, located at residues Asn 222, Asn 264 and Asn 272. Alteration of one or more of these sites is preferred when CPG2 is used in the present invention. Desirably, the alteration is substitution to leucine or glutamine. Such modifications may also be made in the practice of the present invention.

Other variants of naturally occurring sequences may also be made provided such variants retain carboxypeptidase activity. For example, variants with amino acid sequences having at least

50%, preferably 60% more preferably 70%, 80%, 85%, 90% or 95% amino acid identity may be used. Amino acid identity may be determined by standard algorithms available in the art such as the BLAST algorithms using default parameters.

Where variations of the naturally occurring sequences are made these will be such that the enzyme retains it ability to convert the prodrugs of the invention to their corresponding active drugs at substantially the same rate as the unchanged, unglycosylated enzyme. In this context, "substantially unchanged" will desirably be within 1 order of magnitude, and preferably from about 2-fold less to 2 or 5 fold more.

Carboxypeptidase enzymes may be isolated from natural sources or produced recombinantly, for example, as described in WO96/03515.

The carboxypeptidase may be expressed as a fusion with a signal sequence which directs the enzyme to the surface of a cell. Such a system is described in WO96/03515.

Prodrugs of the present invention may also be used as reagents in *in vitro* systems to test the activity of candidate carboxypeptidase enzymes for incorporation into ADEPT, LIDEPT or GDEPT systems or to test cells such as biopsy samples for their suitability for treatment with an enzyme prodrug system.

Such model systems may also be used to test the toxicity, activity or selectivity of compounds of the present invention against a range of tumour cell types. Suitable tumour cells for use in these model systems include ovarian, colon, glioma, breast, small cell and non-small cell lung cancer cells, and

melanomas.

In a further aspect, the present invention provides a pharmaceutical composition, medicament, drug or other composition comprising a prodrug of the invention. The composition may include a pharmaceutically acceptable carrier or diluent.

The invention also provides a kit comprising:

(a) a prodrug of the invention; and, one of:

(b(i)) an immunoglobulin/enzyme fusion protein or conjugate in which the immunoglobulin is specific for a cellular (e.g. tumour-associated) antigen and the enzyme is a carboxypeptidase enzyme;

(b(ii)) a ligand-enzyme conjugate or fusion protein, the ligand being specific for a cellular (e.g. tumour associated antigen) and the enzyme is a carboxypeptidase enzyme;

(b(iii)) a vector which encodes a carboxypeptidase enzyme which can be expressed in a cell (e.g. tumour cell).

In the kits of the invention, the vectors conjugates or fusion proteins may themselves be provided in a composition including a pharmaceutically acceptable carrier or diluent.

Compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise mature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or

intravenous.

For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient may be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection or Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

In ADEPT, the prodrug will normally be administered parenterally, e.g. intravenously or intraperitoneally. Thus, the pharmaceutical composition of the invention may be one which is suitable for parenteral administration. Such a composition conveniently contains the prodrug and isotonic saline or bicarbonate as diluent.

GDEPT will normally involve parenteral administration of both the prodrug and the vector encoding the carboxypeptidase enzyme. Administration by the intravenous route is frequently found to be the most practical. For glioblastoma the route is often intratumoural.

Liposomes containing the vector may be administered by injection in a sterile solution. Viruses may be isolated from packaging cell lines and may be administered by regional perfusion or direct intratumoural injection, or direct injection into a body cavity (intracaviterial administration), for example by intra-peritoneum injection.

Compositions according to the present invention, and for use in

accordance with the present invention, may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water, for injections, immediately prior to use. Injection solutions and suspensions may be prepared extemporaneously from sterile powders, granules and tablets of the kind previously described.

Administration of the prodrug and/or vector and/or fusion and/or conjugate is preferably in a "therapeutically effective amount", that being sufficient to show benefit to the patient.

The doses of each component and the route and time-course of their administration will ultimately be at the discretion of the physician, who will take into account such factors as the nature and severity of what is being treated and the age, weight and condition of the patient.

Suitable doses of prodrug and conjugate for the ADEPT approach are given in Bagshawe et al. Antibody, Immunoconjugates, and Radiopharmaceuticals (1991), 4, 915-922. A suitable dose of conjugate may be from 2000 to 200,000 enzyme units/m^2 (e.g. 20,000 enzyme units/m^2) and a suitable dose of prodrug may be from 20 to 2000 mg/m^2 (e.g. 200 mg/m^2).

In order to secure maximum concentration of the fusion protein or conjugate at the site of desired treatment, it is normally desirable to space apart administration of the two components by at least 4 hours. The exact regime will be influenced by various factors including the nature of the tumour to be targeted and the exact nature of the prodrug. A typical regime is to administer the conjugate at 0 h, galactosylated clearing antibody at 24 h, and prodrug at 48 h. If no clearing antibody

is used, it would generally be longer than 48 h before the prodrug could be injected.

In using the LIDEPT systems of the present invention the prodrug will usually be administered following administration of the ligand-enzyme fusion protein or conjigate. Typically, the ligand/enzyme will be administered to the patient, and its uptake monitored, for example by recovery and analysis of a biopsy sample of targeted tissue or by injecting trace-labelled protein ligand enzyme.

In using the GDEPT system the prodrug may be administered following administration of the vector encoding the activating enzyme. Typically, the vector will be administered to the patient and then the uptake of the vector by transfected or infected (in the case of viral vectors) cells monitored, for example by recovery and analysis of a biopsy sample of targeted tissue.

The amount of vector delivered will be such as to provide an effective cellular concentration of enzyme so that the prodrug may be activated in sufficient concentration at the site of a tumour to achieve a therapeutic effect, e.g. reduction in the tumour size. This may be determined by clinical trials which involve administering a range of trial doses to a patient and measuring the degree of infection or transfection of a target cell or tumour. The amount of prodrug required will be similar to or greater than that for ADEPT systems of the type mentioned above.

A treatment according to the present invention may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

The following examples serve to illustrate the invention. The following reaction schemes summarise the processes of the examples:

EXAMPLE 1

(i) 3.5-Difluoro-4-[bis(2-hydroxyethyl)amino]benzonitrile

A solution of 3,4,5-trifluorobenzonitrile (5g, 32mmol) and diethanolamine (8ml, 85mmol) in N,N-dimethylacetamide was stirred for 10 days. The solvent was then evaporated, the residue partitioned between CH_2Cl_2 (200ml) and H_2O (200ml), the organic layer dried ($MgSO_4$) and evaporated to dryness. The residue was chromatographed using CH_2Cl_2- EtOAc as eluant to give, after recrystallization from toluene, 2.7g (35%) of pure white crystals: mp 63-65°C; ^1H NMR δ 3.33 (t, 4H, NCH_2, J = 6Hz), 3.48 (t, 4H, OCH_2, J = 6Hz), 4.43 (bs, 2H, OH), 7.57 (dd, 2H, H2+6, $J_{H2,\ F3\ and\ H6,\ F5}$ = 8.5Hz, $J_{H2,\ F5\ and\ H6,\ F3\ =\ 2Hz}$ = 2Hz); ^{19}F NMR δ -116.0 (d, 2F, F3+5, $J_{F3,\ H2\ and\ F5,\ 6}$ = 7.5Hz); MS m/z 265 ($M+Na^+$, 10), 243 ($M+H^+$, 100), 211 ($M-CH_2OH$, 85). Anal. ($C_{11}H_{12}N_2O_2F_2$) C,H,N.

(ii) 3.5-Difluoro-4-[bis(2-hydroxyethyl)amino]benzoic acid

A solution of the 3.5-Difluoro-4-[bis(2-hydroxyethyl) amino]benzonitrile (3.15g, 13mmol) and NaOH (5.2g, 0.13mol) in aqueous ethanol (65ml, 50%) was refluxed for 2.5 hours. The solution was then partitioned between EtOAc (800ml) and HCl (330ml, 0.4M), the aqueous layer washed with EtOAc (3x150ml), the combined organic layers dried ($MgSO_4$) and evaporated to dryness. The solid recrystallized from EtOAc (100ml) to give 2.94g (87%) of pure white crystals: mp 146.5- 150°C; ^1H NMR δ 3.3 (t, 4H, CH_2N), 3.46 (q, 4H, CH_2O, J = 5.5Hz), 4.49 (t, 2H, OH, J = 5Hz), 7.44 (d, 2H, H2+6, J = 10.5Hz), 13.1 (bs, 1H,

CO_2H); ^{19}F NMR δ -117.3 (d, 2F, F3+5, J = 9.5Hz); MS m/z 284 ($M+Na^+$, 15), 262 ($M+H^+$, 100), 230 ($M-CH_2OH$, 40). Anal. ($C_{11}H_{13}NO_4F_2$) C,H,N.

(iii) Di-*tert*-butyl [3.5-difluoro-4-[bis(2-iodoethyl)amino] benzoyl]-L-glutamate

a) To a solution of di-*tert*-butyl-L-glutamate hydrochloride (2.66g, 9.0mmol) in dry dimethylformamide (135ml) was added Et_3N (2.5ml, 18mmol), the 3,5-Difluoro-4-[bis(2-hydroxyethyl)amino]benzoic acid (2.35g, 9.0mmol) followed by diethylcyanophosphonate (1.5ml, 9.9ml). After stirring for 3 days the solvent was evaporated and the residue partitioned between EtOAc (450ml) and H_2O (375ml). The organic layer was washed with citric acid (180ml, 10%), sat. sodium bicarbonate solution (180ml), dried ($MgSO_4$) and evaporated to dryness, giving 5.6g of the bis-hydroxy compound.

b) To a solution of the bis-hydroxy compound in dry CH_2Cl_2 (165ml) was added 4-dimethylaminopyridine (0.22g, 1.8mmol) and Et_3N (6.3ml, 45mmol). This solution was cooled in ice, methane sulphonic anhydride (6.3g, 36mmol) dissolved in dry CH_2Cl_2 was added over a few minutes and the reaction was allowed to warm up to room temperature. After 16 hr CH_2Cl_2 (150ml) was added, the solution extracted with 10% aqueous citric acid (375ml), dried ($MgSO_4$) and evaporated to dryness to give the bis-mesyl compound as a brown oil.

c) A solution of the bis-mesyl and NaI (13.5g, 0.09mol) in acetone (150ml) was refluxed for 5 hr. The solvent was removed by evaporation, the residue partitioned between CH_2Cl_2 (375ml) and H_2O (375ml), the organic layer dried ($MgSO_4$) and evaporated to dryness. The impure product was purified on silica using CH_2Cl_2 as eluant giving 5.6g (86%) of pure bis-iodo ester. ^1H

NMR δ 1.39 + 1.41 (2s, 18H, *tert*-Bu), 2.0 (m, 2H, CH_2CH), 2.33 (t, 2H, CH_2CO), 3.3 (t, 4H, CH_2I), 3.57 (t, 4H, CH_2N, J = 7Hz), 4.3 (m, 1H, CH), 7.6 (d, 2H, H2+6, J = 10Hz), 8.60 (d, 1H, NH, J = 7.5Hz); ^{19}F NMR δ -117.2 (d, 2F, F3+5, J = 10Hz).

(iv) [3.5-Difluoro-4-[bis(2-iodoethyl)amino]benzoyl]-L-glutamic acid

The bis-iodo ester (5.6g, 7.8mmol) was dissolved in TFA (140ml). After one hour the solvent was removed by evaporation and the residue evaporated six times with EtOAc to remove TFA. The oil was redissolved in EtOAc, toluene was added and the solvent partly evaporated. The acid crystallized as a pure white solid (4.5g, 95%): mp 121-123°C; ^1H NMR δ 1.95 + 2.05 (2m, 2H, CH_2CH), 2.34 (t, 2H, CH_2CO, J = 7.5Hz), 3.3 (t, 4H, CH_2I), 3.57 (t, 4H, CH_2N, J = 7Hz), 4.4 (m, 1H, CH), 7.6 (d, 2H, H2+6, J = 10Hz), 8.6 (d, 1H, NH, J = 7.5Hz), 12.5 (bs, 1H, CO_2H); ^{19}F NMR δ -117.2 (d, 2F, F3+5, J = 10Hz); MS m/z 633 ($M+Na^+$, 23), 611 ($M+H^+$, 40), 464 (M-glu, 82). Anal. ($C_{16}H_{18}N_2O_5F_2I_2$) C,H,N.

Example 2

[3.5-Difluoro-4-[bis(2-chloroethyl)amino]benzoyl]-L-glutamic acid

The bis-mesyl compound of example 1(iii)(c), 2mmol, was dissolved in N,N-dimethyl acetamide (35 ml) containing LiCl (0.85g, 20mmol) and heated at 85°C for 45 minutes. The solvent was removed by evaporation and the residue partitioned between CH_2Cl_2 (100ml) and H_2O (30ml). The organic layer was dried ($MgSO_4$), evaporated to dryness and the residue chromatographed using CH_2Cl_2 as eluant to give 1.11g of product.

The product was dissolved in 28 ml TFA and treated as in

Example 1(iv) to provide 0.88 g of crystalline product.

^1H NMR δ 1.95 + 2.05 (2m, 2H, CH_2CH), 2.35 (t, 2H, CH_2CO_2, J = 7.5Hz), 3.57 (t, 4H, CH_2N, J = 6Hz), 3.68 (t, 4H, CH_2Cl, J = 6Hz), 4.4 (m, 1H, CH), 7.60 (d, 2H, H2+6, J = 10Hz), 8.61 (d, 1H, NH, J = 7.5Hz); ^{19}F NMR δ -117.44 (d, 2F, F3+5, J = 10Hz); MS m/z 449 ($M+Na^+$, 17), 427 ($M+H^+$, 51), 377 ($M-CH_2Cl$, 42), 280 (M-glu, 100). ($C_{16}H_{18}N_2O_5F_2Cl_2$.0.5toluene) CHN.

Example 3

[3.5-Difluoro-4-[bis(2-bromoethyl)amino]benzoyl]-L-glutamic acid

The bis-mesyl compound of example 1(iii)(c), 0.9 mmol, was dissolved in acetone (15 ml) containing LiBr (0.75 g, 9 mmol) and refluxed for 5 hours. The solvent was removed by evaporation and the residue partitioned between CH_2Cl_2 (30 ml) and H_2O (15 ml). The organic layer was dried ($MgSO_4$)Φ, and evaporated to dryness. The residue was chromatographed using CH_2Cl_2-EtOAc as eluant to give 0.4 g of product.

The product was dissolved in 10 ml TFA and treated as in Example 1(iv) to provide 0.32 g of crystalline product.

^1H NMR δ 1.95 + 2.05 (2m, 2H, CH_2CH), 2.35 (t, 2H, CH_2CO_2, J = 7.5Hz), 3.55 (t, 4H, CH_2N, J = 5.5Hz), 3.61 (t, 4H, CH_2Br, J = 5.5Hz),
4.4 (1H, m, CH), 7.60 (d, 2H, H2+6, J = 10Hz), 8.62 (d, 1H, NH, J = 7.5Hz); ^{19}F NMR δ -117.32 (d, 2F, F3+5, J = 10Hz); MS m/z 539 ($M+Na^+$, 24), 517 ($M+H^+$, 59), 423 ($M-CH_2Br$, 33), 370 (M-

glu, 100). Anal.
($C_{16}H_{18}N_2O_5F_3Cl_2$.0.32toluene) CHN.

EXAMPLE 4

(i) Di-*tert*-butyl 2,3,4,5-tetrafluorobenzoyl-L-glutamate

To an ice-cold solution of di-*tert*-butyl L-glutamate hydrochloride (6.6g, 22.4mmol) and Et_3N (6.9ml, 50mmol) in dry CH_2Cl_2 (70ml) was added, over a period of 1.5 hr, 2,3,4,5-tetrafluorobenzoyl chloride (5.0g, 23.5mmol) in dry CH_2Cl_2 (20ml) and thirty minutes later the solution was allowed to warm to room temperature. Sixteen hours later the solution was washed with H_2O, dried ($MgSO_4$) and evaporated to dryness. ^{1}H NMR δ 1.40 + 1.42 (2s, 18H, *t*-butyl), 1.85 + 1.95 (2m, 2H, *CH_2*CH), 2.34 (t, 2H, CH2CO, J = 7.5Hz), 4.3 (m, 1H, CH), 7.57 (m, 1H, H6), 8.80 (d, 1H, NH, J = 7.5Hz); ^{19}F NMR δ -138.6 + 139.0 (2m, 2F, F2+5), -152.4 (m, 1H, F4), -154.9 (t, 1F, F3, J = 20Hz).

(ii) Di-*tert*-butyl [4-[bis(2-hydroxyethyl)amino]-2,3,5-trifluorobenzoyl]-L-glutamate

A solution of the tetrafluoroamide (4.35g, 10mmol) and diethanolamine (3.0ml, 30mmol) in N,N-dimethylacetamide (50ml) was heated at 130°C with stirring for 2.5 days. The solvent was removed by evaporation and the residue partitioned between CH_2Cl_2 (150ml) and H_2O (150ml). The organic layer was dried ($MgSO_4$), evaporated to dryness and chromatographed using

CH_2Cl_2-EtOAc as eluant to give 2.7g (52%) of product. ^1H NMR 1.39 + 1.41 (2s, 18H, *t*-butyl), 1.95 (m, 2H, CH_2CH), 2.32 (t, 2H, CH_2CO_2, J = 7.5Hz), 3.3 (t, 4H, CH_2N), 3.49 (q, 4H, CH_2O, J = 5.5Hz), 4.3 (m, 1H, CH), 4.52 (t, 2H, OH, J = 5Hz), 7.18 (q, 1H, H-6), 8.48 (d, 1H, NH, J = 7.5Hz); ^{19}F NMR δ -122.7 (t, 1F, F5), -142.0 (m, 2F, F2+3).

(iii) Di-*tert*-butyl [4-[bis(2-chloroethyl)amino]-2,3,5-trifluorobenzoyl]-L-glutamate

a) A solution of the bis-hydroxy compound (2.6g, 5.0mmol), 4-dimethylaminopyridine (90mg, 0.7mmol) and Et_3N (2.5ml, 17.5mmol) in dry CH_2Cl_2 (70ml) was cooled in ice. Methane sulphonic anhydride (2.5g, 14mmol), dissolved in dry CH_2Cl_2 (20ml), was added over two minutes and the solution was allowed to warm up to room temperature. After 16 hr CH_2Cl_2 (100ml) was added and the solution was extracted with 10% aqueous citric acid, dried ($MgSO_4$) and evaporated to dryness to give an oil (3.8g).

b) The bis-mesyl compound was dissolved in N,N-dimethylacetamide (25ml) containing LiCl (0.5g, 10mmol). After 16 hr the solvent was removed by evaporation and the residue partitioned between CH_2Cl_2 (50ml) and H_2O (20ml). The organic layer was dried ($MgSO_4$), evaporated to dryness and the residue chromatographed using CH_2Cl_2 as eluant to give 1.35g (97%) of colourless oil.

(iv) 4-[Bis(2-chloroethyl)amino]-2,3,5-trifluorobenzoyl-L-glutamic acid

The bis-chloro ester (1.27g, 2.3mmol) was dissolved in TFA (30ml). After one hour the solvent was evaporated and the residue evaporated five times with EtOAc. The product was redissolved in EtOAc, toluene was added and the solution evaporated to low volume. More toluene was added and the solution partially evaporated again giving a large mass of white crystals (1.015g, 97.5%): mp 110°C; ^{1}H NMR δ 1.9 + 2.05 (2m, 2H, CH_2CH), 2.34 (t, 2H, CH_2CO, J = 7.5Hz), 3.60 (t, 4H, CH_2N, J = 6HZ), 3.71 (t, 4H, CH_2Cl, J = 6HZ), 4.4 (m, 1H, CH), 7.25 (m, 1H, H6), 8.56 (d, 1H, NH, J = 8Hz); ^{19}F NMR δ -122.67 (t, 1F, F5, $J_{F2,\ 5}$ = 13.5Hz), -141.3 (m, 1F, F2), -141.5 (d, 1F, F3, $J_{F2,\ 3}$ = 19Hz); MS m/z 467 (M+Na^+, 40), 445 (M+H^+, 82), 395 (M-CH_2Cl, 41), 298 (M-glu, 100). Anal. ($C_{18}H_{17}N_2O_5F_3Cl_2$0.08toluene) C,H,N.

Example 5

Di-*tert*-butyl [4-[bis(2-bromoethyl)amino]-2,3,5-trifluorobenzoyl]-L-glutamate

The bis-mesyl compound of example 4(iii)(b), 3 mmol, was dissolved in acetone (25 ml) containing LiBr (0.87 g, 10 mmol) and refluxed for 5 hours. The solvent was removed by evaporation and the residue partitioned between CH_2Cl_2 (50 ml) and H_2O (20 ml). The organic layer was dried ($MgSO_4$),

evaporated to dryness and the residue chromatographed using CH_2Cl_2 as eluant to give 1.33 g of product.

The product was dissolved in 30 ml TFA and treated as in Example 4(iv) to provide 1.0 g of crystalline product.

^{1}H NMR δ 1.90 + 2.05 (2m, 2H, *CH_2*CH), 2.35 (t, 2H, CH_2CO_2, J = 7.5Hz), 3.58 (t, 2H, CH_2N, J = 5.5Hz), 3.66 (t, 4H, CH_2Br, J = 5.5Hz), 4.4 (m, 1H, CH), 7.25 (dd, 1H, H6, $J_{H5, F5}$ = 12Hz, $J_{H6, F3}$ = 5.5Hz), 8.56 (d, 1H, NH, J = 7.5Hz); ^{19}F NMR δ -122.4 (m, 1F, F5), -141.5 (m, 2F, F2+3); MS m/z 557 ($M+Na^+$, 31), 535 ($M+H^+$, 70), 455 (M-Br, 14), 441 (M-CH_2Br, 30), 388 (M-glu, 100). Anal. ($C_{16}H_{17}N_2O_5F_3Br_2$.0.06toluene) C,H,N.

Example 6

Di-*tert*-butyl [4-[bis(2-iodoethyl)amino]-2,3,5-trifluorobenzoyl]-L-glutamate

The bis-mesyl compound of example 4(iii)(b), 1 mmol, was dissolved in acetone (15 ml) containing NaI (1.5 g, 10 mmol) and refluxed for 5 hours. The solvent was removed by evaporation and the residue partitioned between CH_2Cl_2 (45 ml) and H_2O (15 ml). The organic layer was dried ($MgSO_4$), evaporated to dryness and the residue chromatographed on silica using CH_2Cl_2 as eluant to give 0.7 g of product.

The product was dissolved in 17 ml TFA and treated as in Example 4(iv) to provide 0.53 g of crystalline product.

^1H NMR δ 1.9 + 2.05 (2m, 2H, CH_2CH), 2.34 (t, 2H, CH_2CO_2, J = 7.5Hz), 3.3 (t, 4H, CH_2I), 3.59 (t, 4H, CH_2N, J = 7Hz), 4.38 (m, 1H, CH), 7.28 (dd, 1H, H6, $J_{H6, F5}$ = 12Hz, $J_{H6, F3}$ = 2.5Hz), 8.52 (d, 1H, NH, J = 7.5Hz); ^{19}F NMR -122.7 (t, 1F, F5, $J_{F5, H6}$ = 14Hz), -141.3 (m, 2F, F2+3); MS m/z 651 ($M+Na^+$, 23), 629 (M+H+, 65), 482 (M-glu, 100). Anal. ($C_{16}H_{17}N_2O_5F_3I_2$.0.05toluene) C,H,N.

Example 7.

The compound of Example 1 was assessed in a Nu/Nu BalbC mouse bearing a breast carcinoma xenograft that comprised 50% cells stably expressing β-galactosidase plus 50% cell stably expressing a surface tethered CPG2 mutant enzyme (STCPG2(Q)3) (see WO96/03515). This was compared to the prior art compound (3-fluoro-4-[bis(2-chloroethyl)amino]benzoyl]-L-glutamic acid. The controls comprised mice bearing the same tumour xenograft, that were not treated with prodrug.

The results are shown in Figures 1A and 1B. As is seen, treatment with 600mg/kg of the compound of Example 1 (Figure 1A) provided a 50% survival rate for over 200 days, whereas the untreated control group did not survive for more than 40 days.

With the prior art compound (Figure 1B) the survival rate of an untreated control group was comparable to the untreated group shown in Figure 1A, and although treatment provided a measurable effect, and 50% survival was found up to about 70 days post-treatment, it should be noted that to achieve this a

higher dose (1200mg/kg) of the prodrug was required. This translates on a molar basis to 0.98mM for Example 1 (iv) and 2.9mM for the prior art compound.

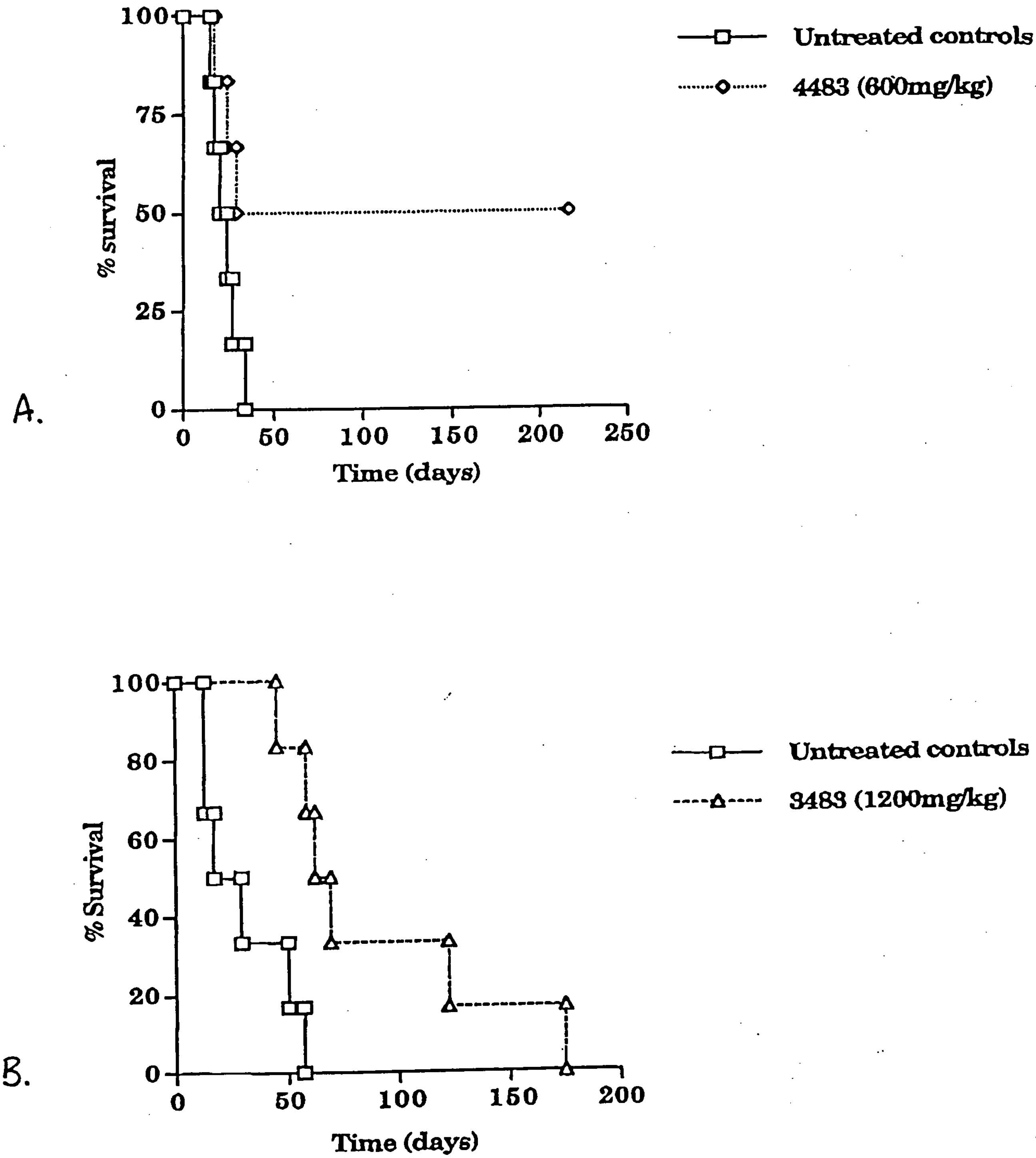

A.
100
75
50
25
0
% survival
0
50
100
150
200
250
Time (days)
Untreated controls
4483 (600mg/kg)
B.
100
80
60
40
20
0
% Survival
0
50
100
150
200
Time (days)
Untreated controls
3483 (1200mg/kg)

FIG. 1

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Form 23/77 : 29.3.00

Agent : Mewburn Ellis